OPTIMIZATION FOR HIGH-LEVEL EXPRESSION IN *PICHIA PASTORIS* AND PURIFICATION OF TRUNCATED AND FULL LENGTH RECOMBINANT SAG2 OF *TOXOPLASMA GONDII* FOR DIAGNOSTIC USE

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Abstract. SAG2 is one of the major surface antigens of the intracellular protozoan parasite *Toxoplasma gondii*. In the present study, truncated recombinant SAG2(S) and full length recombinant SAG2(T) of *T. gondii* were optimally produced (~15 mg/liter) in *Pichia pastoris* expression system using BMMY medium at pH 3, 25ºC in 0.5-1% methanol and a time-course of 1-2 days. The recombinant proteins were purified using a commercial gel filtration purification system obtaining ~33% recovery. The purified SAG2(S) and SAG2(T) showed molecular masses of 45 and 36 kDa by SDS-PAGE, respectively. The recombinant proteins were evaluated by Western blotting with patients' sera and demonstrated 90% sensitivity and 100% specificity for detection of toxoplasmosis. This study provided a means for large-scale expression and purification of SAG2, which should be useful for diagnosis of toxoplasmosis.

Key words: *Toxoplasma gondii*, *Pichia pastoris*, recombinant antigen, surface antigen, expression

INTRODUCTION

Toxoplasmosis is a widespread disease caused by the obligate intracellular parasite *Toxoplasma gondii*. Almost one-third of the human population is infected with *T. gondii* (Bhopale, 2003). Although toxoplasmosis is generally asymptomatic in healthy individuals, the disease may provoke severe problems (encephalitis, pneumonia and disseminated infection) in immunodeficient individuals and neonatal malformations or fetal death in pregnant woman (Hill and Dubey, 2002).

Early diagnosis is pertinent in helping to manage patients who present clinical symptoms of toxoplasmosis. Accurate and definitive serologic diagnosis of recently acquired *T. gondii* infection is still difficult. Current commercially available diagnosis kits are expensive and can be less specific because most kits use total lysate as antigen. It would be worthwhile to develop inexpensive in-house assays for toxoplasmosis. The main task in such effort is to obtain pure and specific antigens for use in the assays.

In our previous study, truncated and full length SAG2 of *T. gondii* have been successfully cloned and expressed using

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the *Pichia pastoris* yeast expression system (Fong *et al*, 2008). These recombinant antigens possess molecular confirmation which could be recognized by monoclonal and human serum-derived antibodies specific for the native SAG2 (Fong *et al*, 2008; Lau and Fong, 2008).

In this study, the recombinant proteins were subject to large scale expression culture in *P. pastoris* in order to produce higher quantity of the recombinant proteins for diagnostic use. The choice of culture conditions used for *P. pastoris* expression system is an important factor in order to improve the productivity of the recombinant protein. Optimal conditions for expression vary according to the kind of *P. pastoris* strain used or the foreign protein expressed (Files *et al*, 2001; Sinha *et al*, 2003). A number of strategies were used in this study to optimize the production level of a recombinant protein in *P. pastoris* including control of environmental parameters such as temperature, pH, methanol concentration and growth medium. The combination of one or all of these cultivation-level strategies would improve yield with minimum proteolytic degradation. Several strategies of protein purification were also conducted to obtain the maximum yield of the purified protein.

MATERIALS AND METHODS

Optimization of expression condition for recombinant SAG2(T) and SAG2(S)

Recombinant plasmids were constructed as previously described (Fong *et al*, 2008; Lau and Fong, 2008) and transformation into *P. pastoris* was performed according to the manufacturer's protocol of the EasySelect™ *Pichia* Expression kit (Invitrogen, CA). Positive clones of full length recombinant SAG(T) and truncated recombinant SAG2(S) were selected for optimum expression. In order to enhance the expression level of recombinant proteins in *P. pastoris*, the growth conditions in shake flasks such as type of medium (buffered methanol complex medium, BMMY; buffered minimal methanol, BMM and minimal medium, MM), methanol concentration (0.25, 0.5, 1, 1.5, and 2%), pH (3, 4, 5, 6 and 7), temperature (25ºC and 30ºC) and induction time were optimized. A general protocol was used for all experiments except for the parameter that was tested. A single recombinant *P. pastoris* colony was picked and inoculated into 250 ml of buffered complex medium containing glycerol. The culture was grown at selected temperature for 24 hours. Cells were harvested and resuspended in 1 liter of buffered complex medium containing methanol. The culture was allowed to grow for 72 hours. Methanol was added every 24 hours to induce expression of the recombinant SAG2 gene. *P. pastoris* cells culture was collected every 12 hours after methanol induction for protein extraction and analysis. Non-recombinant *P. pastoris* host cells (X-33 strain) and X-33 transformed with parent vector (without insert) were similarly treated and analyzed as negative controls.

Protein extraction and purification

For intracellular expression of SAG2(T), total protein was extracted using lysis buffer and acid-washed glass beads as outlined in the EasySelect™ *Pichia* Expression kit manual. For secreted SAG2(S), recombinant *P. pastoris* cells were removed from growth medium by pelleting and an equal volume of 20% (v/v) trichloroacetic acid was added to the growth medium. The mixture was incubated on ice for 1 hour or overnight at -20ºC. The mixture was then centrifuged at 12,000*g* for 30 minutes to harvest the protein precipitate. The pellet was then washed with

acetone, and centrifuged at 12,000*g* for 5 minutes, followed by air drying at room temperature for 10 minutes. The protein was resuspended in phosphate-buffered saline (PBS).

Protein purification was carried out using the Mini Prep Cell® electroeluter (Bio-Rad, CA), in which fractions were collected for 3 hours at 15 minutes intervals. Protein concentration was determined using Bradford assay.

SDS-PAGE and Western blotting

The harvested proteins were separated by SDS-PAGE and transferred by electroblotting to polyvinylidene difluoride membranes (Bio-Rad, USA). The proteins were probed with human serum (at 1:200 dilution). Bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgM or IgG (Bio-Rad, CA). The recombinant proteins also were evaluated in Western blot assays using sera of patients diagnosed with toxoplasmosis (60 samples), amebiasis (10 samples), cysticercosis (10 samples), filariasis (10 samples), malaria (10 samples) and toxocariasis (2 samples). These samples were obtained from the Diagnostic Laboratory at the Department of Parasitology, University of Malaya.

RESULTS

Total protein extracted from recombinant SAG2(T) *P. pastoris* cell showed the appearance of a novel 36 kDa protein at 12 hours post-induction, and its intensity peaked after 36 hours (data not shown). Similar study was carried out on truncated SAG2(S) and a novel protein of 45 kDa appeared 12 hours after induction in the culture medium and it was still detectable until 60 hours, albeit with a decrease in quantity (data not shown). The culture conditions for production of SAG2(T) and SAG2(S) were studied to improve the yield of these recombinant proteins.

In this study, BMMY medium was found to be optimal for the expression of SAG2(T) and SAG2(S) (Fig 1A). A 1.5 to 2-fold differences in expression level were noted between BMMY and MM media. Difference in biomass (data not shown) and expression was also observed between BMMY and BMM media. The highest yield for SAG2(T) and SAG2(S) was 9 and 11 mg/l, respectively in BMMY medium.

Different volumes of pure methanol were added to the media twice daily instead of once in order to decrease the resulting concentration shifts in the BMMY medium. A methanol feeding of 0.5-1% was optimal for yeast growth and expression of SAG2(T) (9 mg/l) and SAG2(S) (12 mg/l) (Fig 1B). Slightly reduced growth and expression were observed when methanol feeding of below 0.5% or above 2% was used.

To determine the pH at which SAG2(T) and SAG2(S) production were optimal, expression was conducted at pH values ranging from 3-7 (Fig 1C, data for pH 6 and 7 are not shown). The highest SAG2(T) (9.5 mg/l) and SAG2(S) (14.2 mg/l) production was observed at pH 3 after 2 days of induction. In addition, there was a sharp reduction in SAG2(S) production above pH 4.

As shown in Fig 1D, the yield of SAG2(T) (13 mg/l) and SAG2(S) (15 mg/l) production was about 1.6 times higher at 25ºC as compared to that at 30ºC after 4 days. Decrease in temperature generally reduces cell death, which prevents the release of proteases.

Purification using the Mini Prep Cell system recovered ~33% of recombinant proteins. Each fraction was analyzed a SDS-PAGE gel. Fractions that contained

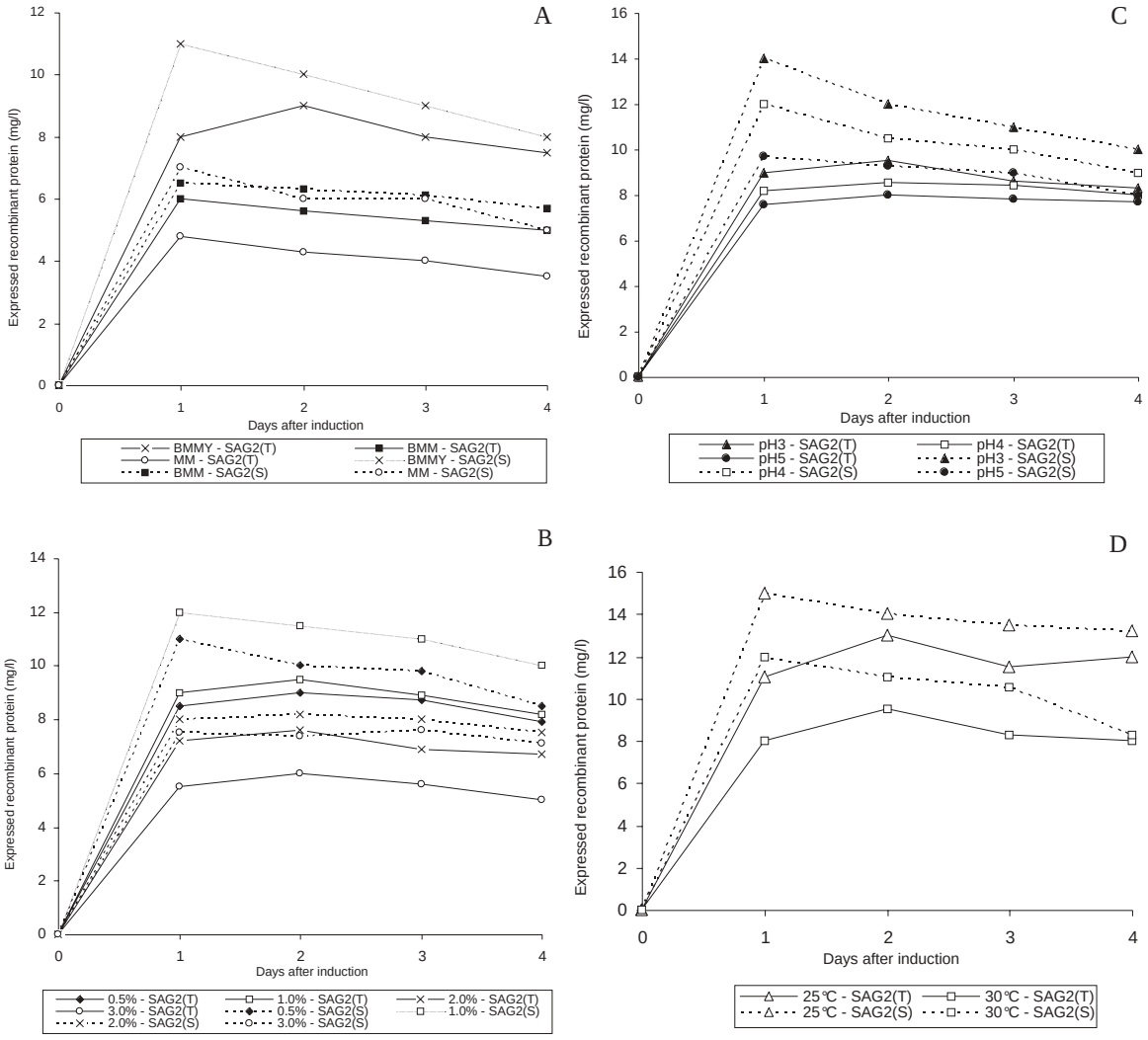

Fig 1–Effects of different media (A), methanol concentration (B), pH (C), and temperature (D) on the expression of recombinant SAG2(T) and SAG2(S). A general protocol was used (culture was grown at selected temperatures for 24 hours. Cells were harvested and resuspended in 1 liter of medium containing methanol. The culture was allowed to grow for 72 hours. Methanol was added every 24 hours to induce expression of the recombinant SAG2 gene), except for the parameter that was tested.

the protein of interest were concentrated. Recombinant SAG2(T) and SAG2(S) could be recognized by 54 out of 60 serum sample of toxoplasmosis patients (data not shown). No cross-reactivity was observed when these recombinant proteins were tested with serum samples from patients infected with other helminths or protozoa (data not shown).

DISCUSSION

Of the 3 media (BMMY, BMM, MM), highest yield for SAG2(T) and SAG2(S)

was in BMMY medium. The components of BMMY, such as peptone, yeast extracts and steady pH had prominent effects on the growth and expression of recombinant proteins in *P. pastoris*. Buffered medium maintains a stable pH condition, and enhances absorption and use of nutrients by the cells. Yeast extracts and peptone contain peptides, amino acids, vitamins and trace elements, which can boost the biomass and energy for protein synthesis during expression (Cregg *et al*, 1993). Product stability is also maintained by the addition of amino acid-rich supplements, which act as alternative and competing substrates for proteases, in addition to repressing protease induction caused by nitrogen limitation (Werten *et al*, 1999).

Methanol taken up by *P. pastoris* cells is oxidized to formaldehyde in a coupled reaction involving alcohol oxidase (AOX) and catalase (CAT) in peroxisomes. These reactions use molecular oxygen as an ultimate electron acceptor. For the expression of protein using *AOX1* promoter, it is important to keep the methanol level within a relatively narrow range. High expression of foreign protein expression depends on rigorous control of methanol level and maintenance of dissolved oxygen in the growth medium. Excess methanol can be toxic to the cell and drastically reduce promoter AOX activity, which may lead to cell death (Brierley *et al*, 1990; Chiruvolu *et al*, 1997; Minning *et al*, 2001). In addition, methanol metabolism requires a high concentration of oxygen and recombinant gene expression is negatively affected by limiting oxygen (Chen *et al*, 1997; Files *et al*, 2001). A methanol feeding of 0.5-1% was optimal for yeast growth and expression of SAG2(T) and SAG2(S). Slightly reduced growth and expression observed when methanol feeding of below 0.5% or above 2% might be due to the limited carbon or the toxic effect of accumulated methanol (Boettner *et al*, 2002).

Although *P. pastoris* can grow in a wide range of pH (3 to 7), pH of 5-6 has been most routinely used for expression in the growth culture (Cregg *et al*, 1993). However, low pH has been shown to significantly increase the yield of recombinant proteins by reducing protease activity (Brierley *et al*, 1994; Koganesawa *et al*, 2002). The highest SAG2(T) and SAG2(S) production observed at pH 3 after 2 days of induction might be due to a decreased production or inactivation of proteases of the *P. pastoris* cells. Shi *et al* (2003) have shown that protease activity in shake flask cultures of *P. pastoris* drastically decreased at pH 3. In addition, there was a sharp reduction in SAG2(S) production above pH 4. At pH 6, the recombinant protein could not be detected, probably due to degradation by high protease activity. Biomass accumulation during the induction phase was measured to determine if the difference in production at different pHs was related biomass accumulation. The result showed that there was no direct correlation between biomass and protein production (data not shown).

The recommended optimal growth and production temperature of *P. pastoris* is 30ºC (according to the instruction manual of *P. pastoris* expression system). In this study, the yield of SAG2(T) and SAG2(S) was higher at 25ºC as compared to that at 30ºC after 4 days. At temperatures above 32ºC protein expression stops and growth decays. High heat loads occur when *P. pastoris* is actively growing or expressing high levels of protein. In actively growing shake flask cultures, it is common for the temperature to increase by 25ºC if left uncontrolled (Chen *et al*, 1997). Yield of recombinant protein was shown to significantly improve at lower

temperatures due to the stability of the recombinant protein (Hong *et al*, 2002). Li *et al* (2000) lowered the temperature of the culture from 30ºC to 23ºC and found a 3-fold increase in the production of recombinant herring antifreeze protein. Jahic *et al* (2003) showed that using a decreasing temperature profile during induction phase can reduce proteolytic degradation of recombinant proteins and increase cell viability.

In this study, attempts to purify the recombinant proteins using polyhistidine-tag purification column by ProBond Purification System were not successful (data not shown). The recovery of protein after purification was less than 0.5 %. Purification was done using the Mini Prep Cell system which is based on molecular weight separation. Purified proteins showed 90% sensitivity and 100% specificity for detection of toxoplasmosis.

In summary, the methylotrophic yeast *P. pastoris* constitutes an efficient expression system for the production of recombinant SAG2(S) and SAG2(T) that are similar to those of the native proteins. Our next step will be to produce recombinant proteins in fermentor, as we have observed through the optimization experiment that initial cell density is a crucial factor for a high level of protein expression. SAG2(S) could be selected for up-scale production as observed in this study that recombinant proteins were efficiently secreted by *P. pastoris*, producing higher yield compare to intracellular expression with properties similar to the native one.

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